Immunohistochemical detection of a substance resembling growth hormone-releasing factor in the brain of the rainbow trout (*Salmo gairdneri*)

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Received 4 January 1989; accepted 22 February 1989

Summary. We studied the distribution of an immunoreactive substance resembling growth hormone-releasing factor (GRF) in the hypothalamus and pituitary gland of the rainbow trout by immunofluorescence methods. The GRF-like immunoreactive perikaryon was observed in colchicine-treated fish. The majority of GRF-containing neurons were located in the nucleus lateral tuberis; others were located in the caudal part of the preoptic nucleus of the hypothalamus. The GRF-like immunoreactive neuronal processes projected into the pars distalis via the pars nervosa of the pituitary gland. The distribution of the GRF-like immunoreactive substance in the hypothalamus and pituitary gland suggests that GRF plays a physiological role in the regulation of growth hormone release from the pituitary gland of rainbow trout, as it does in mammals.
Key words. Growth hormone-releasing factor; immunofluorescence; nucleus lateral tuberis; preoptic nucleus; pars distalis.

Growth hormone-releasing factor (GRF) was first characterized from a human pancreatic islet tumour [1]; however, GRF is primarily produced from the hypothalamus of the brain in most mammalian species [2]. GRF is one of two hypothalamic factors, together with somatostatin (SST) (known as an inhibitor), which control growth hormone (GH) release from the pituitary gland [2]. Immunohistochemically, GRF-like perikarya in the brain of mammals are located mainly in the medial basal hypothalamus [3, 4], and their neuronal terminals are predominantly at the median eminence [5, 6]. This regional distribution of GRF-like immunoreactivity (GRF-LI) in mammals was also demonstrated and quantitatively determined by radioimmunoassay [7, 8]. Ectopical GRF-LI was found in other organs, such as sensory ganglia [9], the pancreas [10], and gastrointestinal tracts [11]. There is little information on GRF for lower vertebrates [12]. An earlier experiment performed in our laboratory using western immunoblots demonstrated the existence of GRF-LI in homogenates of the hypothalamus and pituitary gland of juvenile coho salmon (*Oncorhynchus kisutch*) [13]. Carp GRFs were recently purified by J. Rivier, The Salk Institute, California (pers. comm.). In the present report, we describe the immunohistochemical distribution of GRF-LI substances in the brain of the rainbow trout.

Materials and methods

The studies were carried out on sexually mature, 2-year-old (200–250 g) rainbow trout, *Salmo gairdneri*, purchased from West Creek Trout Farm (Aldergrone, B.C., Canada). Fish were held in 4800-l circular aquaria supplied with flow-through dechlorinated freshwater at an ambient temperature and under a natural photoperiod. Twenty-four hours before being killed, four fish were given bilateral intraventricular injection colchicine (5 µg/5 µl of 0.9% NaCl) under tricaine methane sulfonate (0.04%) anaesthesia, and four were injected with saline to serve as controls. All fish were cannulated via the aorta and fixed by perfusion following the method of Hinton [14]. A rapid injection (10 ml/30 s) of heparin solution (0.01% in 0.9% NaCl) was immediately followed by perfusion of a fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 0.6% picric acid) at a rate of 200 ml in 60–90 min. The brain and pituitary gland were then removed and post fixed in the same fixative for 4 h at 4 °C. After rinsing in 0.1 M phosphate buffer saline (PBS, pH 7.6) for 24 h, the tissues were routinely dehydrated and embedded in paraffin. Sections (6 µm) were serially cut in the frontal or sagittal plane.

Following rehydration, sections were processed for indirect immunofluorescence as originally described by Weller and Coons [15]. The primary antisera used were either rabbit antiserum against synthetic human pancreatic GRF (1–44) (no. 8119-11, kindly supplied by N. Sherwood, University of Victoria, Canada) or rabbit antiserum against rat hypothalamic GRF (1–37) (no. rG75, kindly supplied by J. Rivier, The Salk Institute, California) conjugated to human α-globulin with glutaraldehyde. Briefly, sections were incubated with the primary antisera at dilutions of 1:500–1:1000 in PBS and placed in a humid atmosphere at 4 °C for 24 h. Following two washes with PBS for 10 min each, the sections were then treated with secondary antiserum, goat anti-rabbit IgG (conjugated with FITC [Sigma]) at a concentration of 1:50 diluted in PBS, for 4 h at room temperature. After another 3 rinses in PBS for 10 min each, sections were mounted under a glycerin:PBS (3:1) solution with coverslips before observation with a Carl Zeiss fluorescent microscope.

Specificity controls involved the replacement of the antisera by nonimmune rabbit serum at the same concentration. We also examined the primary antisera preabsorption for 4 or 24 h at 4 °C with hpGRF (1–44) (Sigma), carp GRF (1–45), carp GRF (1–29) (both of the carp GRFs were kindly supplied by J. Rivier), SST (Sigma) and TRH (Sigma) at a concentration of 7.5 nM/ml of

1:1000 diluted antiserum. To eliminate possible crossreaction against carrier proteins, human α-globulin (1 mg/ml, Sigma) was also preincubated with the primary antisera.

Results and discussion

Both the antiserum against rGRF (1–37) and the antiserum against hpGRF (1–44) produced positive GRF-LI. Immunoreactive GRF was present in perikarya, neuronal processes, and terminals in areas of the hypothalamus and pituitary gland of rainbow trout (fig. 2). Perikarya of GRF-LI were located mainly in the ventral and lateral parts of the nucleus lateral tuberis (NLT) of the hypothalamus (fig. 1a). The areas containing positive perikarya were arranged rostrocaudally, almost throughout the NLT, at an approximate distance of 200 µm. There were only about 10–20 GRF-LI neurons with medium-sized cell bodies per section (fig. 1a). The perikarya of GRF-LI were also found in the caudal part of the preoptic nucleus (NPO) with a very small number of neurons (not illustrated). The fluorescence of the perikarya was strong only in fish brains pretreated with colchicine, although GRF-LI neuronal processes and terminals were still clear in control fish. The positive neuronal processes appeared to accumulate in the dorsal part of the pituitary gland (close to the third ventricle) extending inwards a short distance rostrally and caudally (fig. 1c). The GRF-LI fibers were distributed in the pars nervosa in the middle part of the pituitary gland and ended at the pars distalis, mainly terminating at the interface of the pars nervosa and the pars distalis (fig. 1d).

The staining specificity of antisera showed that the positive immunoreaction stained by antiserum against hpGRF (1–44) was blocked when it was preabsorbed with hpGRF (1–44). However, it was only partially blocked by preabsorption of either carp GRF (1–45) or carp GRF (1–29). The positive immunoreaction stained by antiserum against rGRF (1–37) was partially blocked by preabsorption of hpGRF (1–44) and almost abolished by preabsorption of either carp GRF (1–45) or carp GRF (1–29). The preabsorption of SST or TRH did not block the GRF-LI staining by either of the antisera against rGRF (1–37) or hpGRF (1–44). There was no positive staining when sections were incubated with non-immune rabbit serum.

Immunostained perikarya were clearly present in those fish pretreated with intraventricular administration of colchicine. It is well known that neural products, such as neurohormones or neurotransmitters, are synthesized inside the cell body and then transported to the terminal bulb[16]. A variety of evidence suggests that microtubules play an important role in the transport (rapid) of products along the axon[16]. Colchicine is an agent that binds to tubulin to form a tight complex[17], and induces a conformation change during microtubule assembly[18]. Consequently, the microtubule loses its normal function[19], and neural products accumulate in the cell body. The immunoreactive neurons were almost invisible in fish without colchicine treatment, as was shown previously in the rat[20]. However, the dose of colchicine used in these fish was 20 times lower than that in rats of similar weights[20]. For some reason, higher doses caused the fish to die within an hour.

In the rat, the neurons containing GRF-LI were considered to be located predominantly in the arcuate nucleus (ARC) and the dorsal and ventral margins of the ventromedial nucleus (VMN)[3,20]. This distribution was immunohistochemically demonstrated by both polyclonal and monoclonal primary antibodies against rGRF[4]. In primates, including humans, the GRF-LI was distributed similar to that in the rat with the exception that GRF-containing perikarya were present throughout the VMN, not only at the dorsal and ventral portion[21]. The localization of GRF-LI is consistent with the areas localized by stimulation and lesion studies[22]. Apart from these two nuclei, the GRF-LI neurons in mammals were also found in other hypothalamic nuclei or areas, such as the dorsal part of the dorsomedial nucleus (DMN), the lateral and medial tuberal nucleus, the paraventricular nuclei (PVN), the medial forebrain bundle, the zona incerta and the medial perifonical region of the lateral hypothalamus[6,23,24]. In teleosts, the NLT (nucleus lateral tuberis) is equivalent to the ARC in mammals[25]. Some characteristics of the GRF-LI distribution in teleosts, for example the GRF-LI perikarya shown on transverse section and the rostralcaudal extent of positively stained neurons in the NLT, were comparable to that seen in the ARC nucleus of mammals[20]. However, the population of GRF-LI neurons in rainbow trout (sexually mature fish) was smaller than that in adult rats of a similar body weight[4]. These differences might be partially explained from an evolutionary point of view. In mammals the forebrain (the cerebrum and the diencephalon) including the hypothalamus, is highly developed[25]. This evolutionary change in the central nervous system may be characterized, both morphologically and functionally, by the increased number of neurons as well as complexity of synaptic connections.

The GRF-LI neuronal processes in mammals that originate in the ARC and VMN project primarily to the median eminence (ME) and terminate on the capillaries of the primary plexus of the portal system[21,23]. However, in fish, the neurohormones produced in the hypothalamus are transported directly to the secretory cells of the anterior pituitary gland[27]. From our observations, the GRF-LI neuronal processes in rainbow trout extended via the pars nervosa and their terminals projected into the pars distalis. This distribution of GRF-LI processes in the pituitary was in agreement with the immunocytochemical localization of GH secreting cells in the caudal pars distalis reported by Wagner and McKeown[28]. The origin of the cluster of GRF-LI fibers that appeared in the dorsal part of the pituitary facing the third ventricle was unknown.

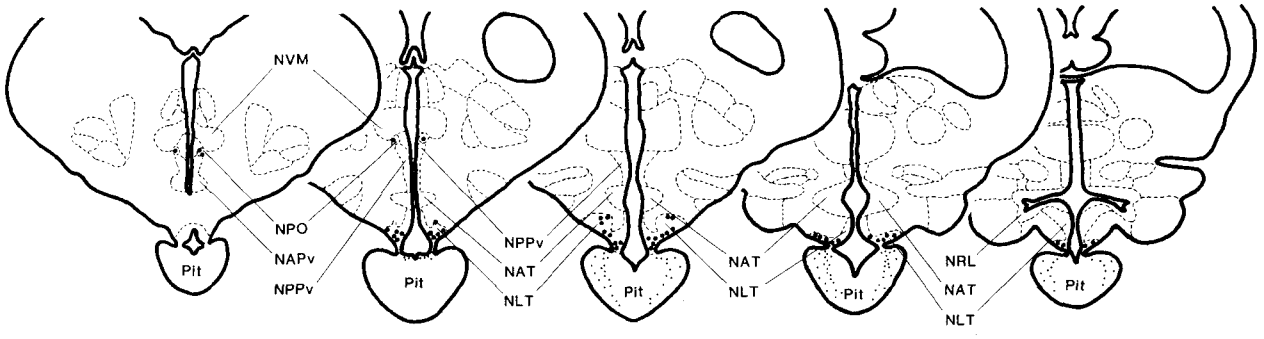

Figure 1. Schematic illustration of frontal sections of the rainbow trout hypothalamus and pituitary gland showing the topography of GRF-like immunoreactive structures. The majority of GRF like immunoreactive cell bodies (large dots) are located in the nucleus lateral tuberis (NLT) of the hypothalamus. Fewer cell bodies are seen in the caudal part of preoptic nucleus (NPO) of the hypothalamus. The GRF-like immunoreactive neuronal processes and terminals (small dots) are distributed in the pars distalis and pars nervosa of the pituitary gland. The sections with positive staining are arranged rostrocaudally at 200 µm distances. The map is from the atlas of R. Billard and R. E. Peter[24]. The abbreviations are as follows: NAPv, nucleus anterioris periventricularis; NAT, nucleus anterior tuberis; NLT, nucleus lateral tuberis; NPO, nucleus preopticus; NPPv, nucleus posterioris periventricularis; NRL, nucleus lateralis; NVM, nucleus ventromedialis thalamic; Pit, pituitary gland.

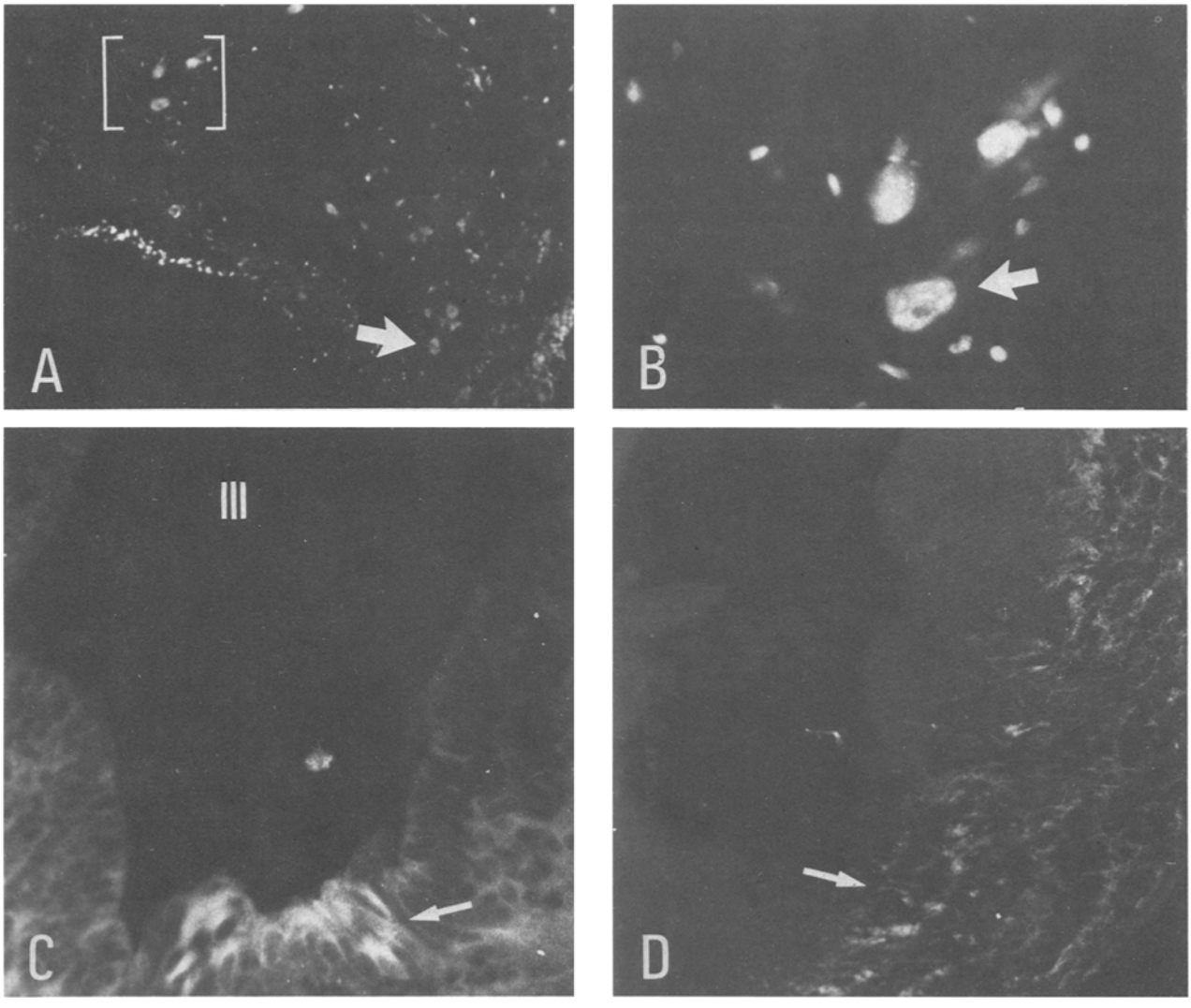

Figure 2. Immunofluorescent micrographs of frontal sections of the basomedial hypothalamus (A, B) and pituitary gland (C, D). *A* Several GRF-like immunoreactive perikarya immunostained with primary antiserum against rGRF (1–37) are present in the nucleus lateral tuberis (NLT) of the hypothalamus of colchicine-treated fish (×310). *B* Higher magnification of rectangle indicated in A (×1160). *C* Abundant neuronal processes immunostained by primary antiserum against hpGRF (1–44) is accumulated at the dorsal part of the pituitary gland facing third ventricle from the fish without colchicine treatment (×800). *D* GRF-like immunoreactive neuronal processes are distributed in the pars nervosa of the pituitary gland and the terminals end in the pars distalis. All treatments were the same as in C (×1250). Large arrows indicate the neurons, and small arrows indicate neuronal processes. III, third ventricle.

The location of GRF-LI neurons in the preoptic nucleus in rainbow trout was consistent with that found in codfish (*Gadus morhua*)[12]. The relation between these two GRF-containing nuclei (NLT, NPO) in fish is unclear. However, immunohistochemical evidence from rats showed that GRF-LI neuronal processes from ARC, VMN and other areas of the hypothalamus reach anteriorly to the preoptic area (POA)[22]. Moreover, the POA is known to be the area containing somatostatinergic neurons[29] and in association with the inhibitory control of GH secretion in rats[22]. This information implies the existence of a complex relationship among hypothalamic nuclei in regulating GH release.

Acknowledgment. The antiserum against rGRF (1–37), carp GRF (1–45), and carp GRF (1–29) were generous gifts from Dr J. Rivier, The Salk Institute, California, and the antiserum against hpGRF (1–44) was kindly supplied by Dr N. Sherwood, University of Victoria, Canada. A special note of thanks to Dr R. C. Fargher for his advice in this work and Dr R. J. Snyder for his comments on the manuscript. This research was supported by a grant to BAM from the National Science and Engineering Research Council of Canada.

1 Rivier, J., Spiess, J., Thorner, M., and Vale, W., Nature *300* (1982) 276.
2 Frohman, L. A., and Jansson, J.-O., Endocr. Rev. *7* (1986) 223.
3 Lechan, R. M., Lin, H. D., Ling, N., Jackson, I. M. D., Jacobson, S., and Reichlin, S., Brain Res. *309* (1984) 55.
4 Bruhn, T. O., Anthony, E. L. P., Wu, P., and Jackson, I. M. D., Brain Res. *424* (1987) 290.
5 Lin, H. D., Bollinger, J., Ling, N., and Reichlin, S., J. clin. Endocr. Metab. *58* (1984) 1197.
6 Merchenthaler, I., Vigh, S., Schally, A. V., and Petrusz, P., Endocrinology *114* (1984) 1082.
7 Leidy, J. W. Jr, and Robbins, R. J., J. clin. Endocr. Metab. *62* (1986) 372.
8 Shibasaki, T., Kiyosawa, Y., Masuda, A., Nakahara, M., Imaki, T., Wakabayashi, I., Demura, H., Shizume, K., and Ling, N., J. clin. Endocr. Metab. *59* (1984) 263.
9 Jozsa, R., Korf, H.-W., and Merchenthaler, I., Cell Tissue Res. *247* (1987) 441.
10 Bosman, F. T., and Assche, C. V., J. Histochem. Cytochem. *32* (1984) 1139.
11 Bruhn, T. O., Mason, R. T., and Vale, W. W., Endocrinology *117* (1985) 1710.
12 Pan, J. X., Lechan, R. M., Lin, H. D., Sohn, J., Reichlin, S., and Jackson, I. M. D., Endocrinology *116* (1985) 1663.
13 Luo, D., and McKeown, B. A., Comp. Biochem. Physiol. (1989) in press
14 Hinton, D. E., J. Fish Res. Board Can. *32* (1975) 416.
15 Weller, T. H., and Coons, A. H., Proc. Soc. exp. Biol. *86* (1954) 789.
16 Karp, G., in: Cell Biology, pp. 744. McGraw-Hill Book Company, New York 1984.
17 Wilson, L., and Bryan, J., Adv. cell. molec. Biol. *3* (1974) 21.
18 Garland, D. L., Biochemistry *17* (1978) 4266.
19 Andreu, J. M., and Timasheff, S. N., Proc. natl Acad. Sci. USA *79* (1982) 6753.
20 Daikoku, S., Kawano, H., Noguchi, M., Nakanishi, J., Tokuzen, M., Chihara, K., and Nagatsu, I., Brain Res. *399* (1986) 250.
21 Bloch, B., Brazeau, P., Ling, N., Bohlen, P., Esch, F., Wehrenberg, W. B., Benoit, R., Bloom, F., and Guillemin, R., Nature *301* (1983) 607.
22 Muller, E. E., Physiol. Rev. *67* (1987) 962.
23 Jacobowitz, D. M., Schulte, H., Chrousos, G. P., and Loriaux, D. L., Peptides *4* (1983) 521.
24 Fellmann, D., Bugnon, C., and Lavry, G. N., Neurosci. Lett. *58* (1985) 91.
25 Billard, R., and Peter, R. E., Reprod. Nutr. Develop. *22* (1982) 1.
26 Mill, P. J., in: Comparative Neurobiology, p. 193. Edward Arnold, London 1982.
27 Gorbman, A., Dickhoff, W. W., Vigna, S. R., Clark, N. B., and Ralph, C. L., in: Comparative Endocrinology, p. 45. John Wiley and Sons Inc., New York 1983.
28 Wagner, G. F., and McKeown, B. A., Cell Tissue Res. *231* (1983) 693.
29 Day, T. A., Oliver, J. R., Menadue, M. F., Davies, B., and Willoughby, J. O., Brain Res. *238* (1982) 55.

0014-4754/89/060577-04$1.50 + 0.20/0

Arylpyridyl-thiosemicarbazones: A new class of anti-juvenile hormones active against Lepidoptera

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Received 2 August 1988; accepted 14 March 1989

Summary. A new class of anti-juvenile hormone agents is described. Active anti-juvenile hormone compounds were either diazine thiosemicarbazones or aryl substituted pyridyl thiosemicarbazones, synthesized from substituted benzaldehydes. While many analogs in these classes showed feeding and growth inhibition in a variety of insects, a select group caused formation of precocious pupal characteristics in *Agrotis ipsilon* (black cutworm) and *Heliothis virescens* (tobacco budworm) and black cuticle and precocious pupae in *Manduca sexta* (tobacco hornworm). They were active only by diet incorporation. The symptoms of precocious development could be reversed by co-administration of a juvenoid. One of the active compounds was shown to inhibit juvenile hormone biosynthesis in vitro by corpora allata of the cockroach *Diploptera punctata*. However, none of the compounds were active inhibitors of purified chicken liver prenyl transferase.
Key words. Thiosemicarbazones; anti-juvenile hormone; insect growth regulator; Lepidoptera; juvenile hormone biosynthesis inhibitor.

The search for new classes of insect growth regulators has been hampered by our lack of knowledge about the insect endocrine system and the paucity of different classes of compounds active in agronomic situations. At present only the benzoylphenylureas, exemplified by diflubenzuron and the newer more potent analog CGA-112913[1],